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APPENDIX A

A family of autocrine growth factors in *Mycobacterium tuberculosis*

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Summary

Mycobacterium tuberculosis **and its close relative,** ***Mycobacterium bovis*** **(BCG) contain five genes whose predicted products resemble Rpf from** ***Micrococcus luteus*****. Rpf is a secreted growth factor, active at picomolar concentrations, which is required for the growth of vegetative cells in minimal media at very low inoculum densities, as well as the resuscitation of dormant cells. We show here that the five cognate proteins from** ***M. tuberculosis*** **have very similar characteristics and properties to those of Rpf. They too stimulate bacterial growth at picomolar (and in some cases, subpicomolar) concentrations. Several lines of evidence indicate that they exert their activity from an extra-cytoplasmic location, suggesting that they are also involved in intercellular signalling. The five** ***M. tuberculosis*** **proteins show cross-species activity against** ***M. luteus*****,** ***Mycobacterium smegmatis*** **and** ***M. bovis*** **(BCG). Actively growing cells of** ***M. bovis*** **(BCG) do not respond to these proteins, whereas bacteria exposed to a prolonged stationary phase do. Affinity-purified antibodies inhibit bacterial growth** ***in vitro*****, suggesting that sequestration of these proteins at the cell surface might provide a means to limit or even prevent bacterial multiplication** ***in vivo*****. The Rpf family of bacterial growth factors may therefore provide novel opportunities for preventing and controlling mycobacterial infections.**

Introduction

Intercellular communication between bacteria has been well documented over the last decade (Kaiser and Losick, 1993; Kell *et al.*, 1995; Salmond *et al.*, 1995; Fuqua and Greenberg, 1998). *N*-acyl-L-homoserine lactones are generally used for cell density-dependent signalling in Gram-negative organisms (Fuqua *et al.*, 1994; 1996). Peptides are more commonly, though not exclusively (Horinouchi and Beppu, 1994; Ohnishi *et al.*, 1999), used for intercellular signalling in Gram-positive organisms (Kleerebezem *et al.*, 1997; Lazazzera and Grossman, 1998). Examples of processes in which peptide-mediated signalling occurs include conjugation in enterococci (Clewell, 1993) and development of competence for genetic transformation and endospore formation in *Bacillus subtilis* (Kaiser and Losick, 1993; Lazazzera and Grossman, 1998).

Individual bacteria are normally considered autonomous, because their growth and multiplication does not apparently depend on the presence of any specific exogenous peptidic or proteinaceous growth factors (Kaprelyants *et al.*, 1994a; Votyakova *et al.*, 1994; Kaprelyants and Kell, 1996; Kell and Young, 2000). This conventional view has been challenged by the recent discovery of a protein called Rpf (*r*esuscitation-*p*romoting *f*actor) that is secreted by growing cells of *Micrococcus luteus* (Mukamolova *et al.*, 1998). Rpf was required at picomolar concentrations for the resuscitation of dormant, 'non-culturable' cells of *M. luteus* and for the growth of small inocula in minimal media. Moreover, extensive washing of actively growing cells of *M. luteus* rendered their further growth dependent on exogenously added Rpf (Mukamolova *et al.*, 1998). It has recently been shown that *rpf* is an essential gene in *M. luteus* (Mukamolova *et al.*, 2002). Rpf therefore has the properties of a proteinaceous bacterial growth factor or cytokine (Callard and Gearing, 1994).

Genes resembling *M. luteus rpf* are widespread throughout the high G + C Gram-positive bacteria, which includes streptomycetes, corynebacteria and mycobacteria (Kell and Young, 2000). The DNA sequence databases currently contain more than 30 members of the *rpf* gene family and most organisms contain several representatives. For example, *Mycobacterium tuberculosis* and its close relative (Behr *et al.*, 1999) *Mycobacterium bovis* contain five *rpf*-like genes.

Tuberculosis, caused by *M. tuberculosis*, now kills more people in the world than any other single bacterial infection and globally, one in three people are believed to harbour a persistent (latent) infection (Bloom and Murray,

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1992; Dye *et al.*, 1999). The phenomenon of persistence has long been recognised (McDermott, 1958; Wayne, 1960; McCune *et al.*, 1966), but it remains poorly understood (Young and Duncan, 1995; Parrish *et al.*, 1998; Wayne and Sohaskey, 2001). It is generally agreed that the immune system plays an important role in preventing net bacterial multiplication (Flynn and Chan, 2001), but other aspects of the biology of the persisting organisms remain controversial. Some evidence suggests that they may be metabolically active (reviewed by Höner zu Bentrup and Russell, 2001) whereas there is also evidence that they have become dormant, or have lost culturability (Wayne, 1960, 1994). The presence of *rpf*-like genes in these mycobacteria raises the possibility that (a lack of) their products may be involved in controlling bacterial growth *in vivo*. Persisting organisms in the latent state may require one or more of these proteins in order to re-activate. To illuminate these suggestions, we have isolated recombinant forms of the five *rpf*-like proteins of *M. tuberculosis* and tested their activities using several different organisms.

Results

Comparison of the five rpf-*like genes of M. tuberculosis*

The predicted products of the five *rpf*-like genes of *M. tuberculosis* share with Rpf a conserved ~70-residue segment (Fig. 1A). Rpf is a secreted protein. Therefore the SignalP and TMMHM servers at the Technical University of Denmark (http://www.cbs.dtu.dk/services/SignalP/ and http://www.cbs.dtu.dk/services/TMHMM-2.0/) were used to determine whether the five Rpf-like proteins of *M. tuberculosis* are also likely to be secreted. Two of them, RpfA (Rv0867c, 407 aa) and RpfD (Rv2389c, 154 aa) were predicted to be secreted proteins – see also Gomez *et al.* (2000). RpfA is a comparatively large protein in which the Rpf-like segment is followed by an extensive series (residues 146–320) of proline + alanine-rich repeats with the consensus sequence APADLAPP. The RpfB protein (Rv1009, 362 aa) has its Rpf-like domain at the C-terminus. RpfB is probably anchored to the outer surface of the cell membrane by an N-terminal prokaryotic membrane lipoprotein lipid attachment site (Prosite PS00013). Residues 1–117 of RpfB share similarity with the N-terminal Mce domain (PF02470) that is found in all six predicted products of the multiple *mce* operons of *M. tuberculosis*, at least one of which (*mce1*, Rv0169) is involved in entry into and survival inside macrophages (Arruda *et al.*, 1993). The status of the remaining two Rpf-like gene products is less clear. Although RpfC (Rv 1884c, 176 aa) is not predicted to contain a *trans*-membrane helix near its N-terminus, a secretory signal sequence was predicted using a neural network (http://www.cbs.dtu.dk/services/SignalP/) trained on Gram-positive signal sequences. RpfE (Rv2450c, 172 aa) has a weakly predicted *trans*-membrane helix close to its N-terminus, whereas the presence of a signal sequence was quite strongly predicted. Thus, some of these five proteins are probably secreted, whereas others may be anchored in the cytoplasmic membrane. In common with Rpf, they all probably have extra-cytoplasmic functions.

The various *rpf*-like genes (Fig. 1B), are scattered about the *M. tuberculosis* genome (Cole *et al.*, 1998). The *rpfA* and *rpfE* genes appear to comprise monocistronic operons, the former lying within a cluster of genes concerned with molybdopteroate biosynthesis. There is a

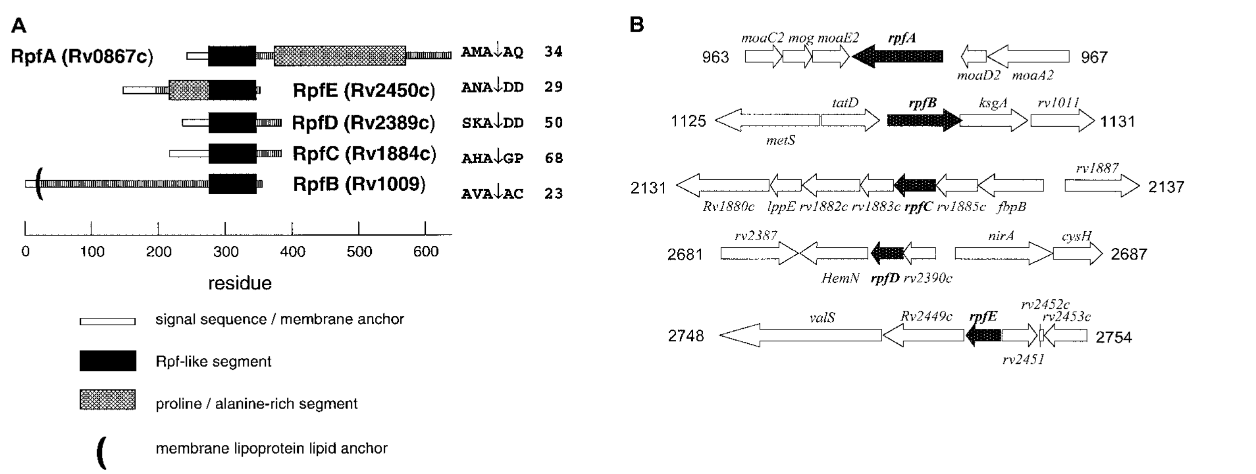

Fig. 1. A. Schematic alignment of the five Rpf-like gene products of *M. tuberculosis*. The predicted (http://www.cbs.dtu.dk/services/SignalP/) signal sequence cleavage sites and the numbers of the first predicted residues of the mature secreted proteins are also indicated.
B. The chromosomal context of each gene (http://genolist.pasteur.fr/TubercuList/). The approximate co-ordinates (kbp) of each segment are also given.

25 bp overlap between the 3′ end of *rpfB* and *ksgA*, which is predicted to encode a dimethyadenosine transferase. The *rpfD* gene lies downstream from a gene of unknown function in what may be a bicistronic operon. It is located between *hemN* and *nirA*, which probably encode proteins involved in coproporphyrinogen III decarboxylation and nitrate reduction respectively. Finally, *rpfC* is the third gene in a seven-gene operon containing a mycolyltransferase (*fbpB*) upstream and a probable dehydrogenase (Rv 1882c), lipoprotein (Rv 1881c) and cytochrome P450 (Rv 1880c) downstream. The widely differing contexts of the five genes provide no clear evidence for a common biological function.

Biological activities of the recombinant proteins

To investigate their biological functions, the five Rpf-like proteins encoded by the *M. tuberculosis* genome were obtained in reagent quantities as polyhistidine-tagged derivatives, lacking their predicted signal sequences/membrane anchors (see Fig. 1A), as indicated in the *Experimental procedures*. They were assayed by incorporating them into the growth medium of *M. luteus* and *M. smegmatis*. We have previously shown that when these fast growing organisms are inoculated at low cell density into a minimal medium, their apparent lag phase (time to detectable turbidity) is reduced in response to Rpf addition (Mukamolova *et al.*, 1998). All four *M. tuberculosis* proteins tested also reduced the apparent lag phase of *M. luteus* (Fig. 2A). RpfA and RpfC were the most potent. The former showed activity at fM concentrations, whereas the latter caused the greatest reduction in apparent lag phase (from 216 h to 80 h). Similarly, all four proteins were active in reducing the apparent lag phase of *M. smegmatis* (Fig. 2B). At optimally active concentrations, all four proteins reduced the apparent lag phase to the same extent and for RpfA, RpfC and RpfE, maximal activity was observed at subpicomolar concentrations (Fig. 2B).

Complete activity profiles were obtained for RpfD against both organisms and for RpfA and RpfC against *M. luteus*. These profiles indicate that there is an optimal concentration range for activity, above and below which there is reduced activity or no activity at all. Indeed, the only protein for which there is no evidence of reduced activity at elevated (μM) concentrations, was RpfE, when tested using *M. luteus*.

The four proteins show different potency profiles when tested using these two fast-growing organisms. RpfA was active at subpicomolar concentrations against both *M. smegmatis* and *M. luteus*. On the other hand, the potency of RpfE was high when tested against *M. smegmatis*, but comparatively low when tested against *M. luteus*. Freshly purified samples were always used for experiments, because these proteins lose biological activity during storage (*Experimental procedures*). Nevertheless, we cannot rule out the possibility that differences in the proportion of biologically active molecules in different protein samples could account, at least in part, for the different potencies of the four proteins. However, this cannot explain the different potency of RpfA when tested with the two different organisms, because the assays were done at the same time with the same protein preparation.

Mycobacterium bovis (BCG) was chosen as a representative of the slow-growing mycobacteria (Wayne, 1984) with which to test the biological activities of the Rpf-like proteins of *M. tuberculosis*. *Mycobacterium bovis* (BCG) is closely related to *M. tuberculosis* (Behr *et al.*, 1999) and contains five *rpf* homologues that are very similar indeed to those of *M. tuberculosis* (Kell and Young, 2000).

The response of *M. bovis* (BCG) to the five proteins

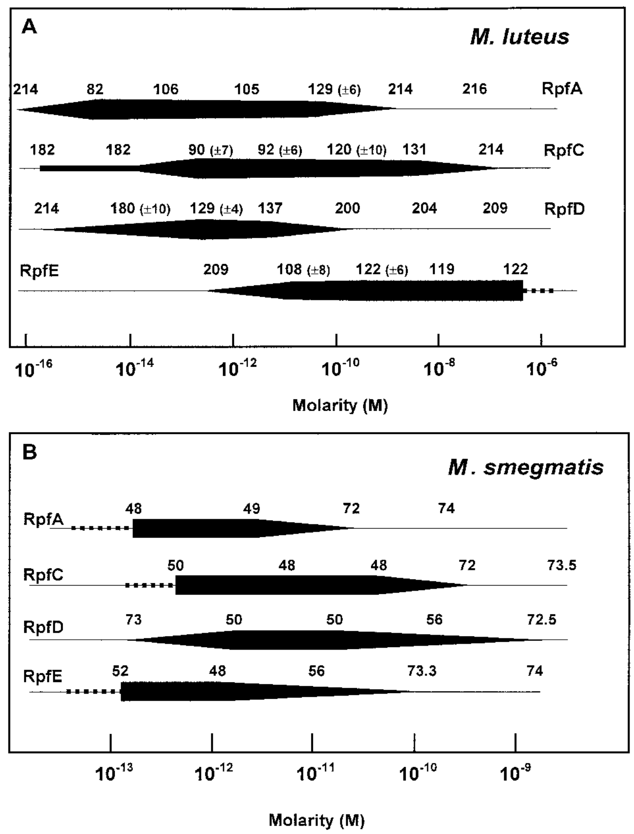

Fig. 2. The Rpf-like proteins of *M. tuberculosis* reduce the apparent lag phase of *M. luteus* (A) and *M. smegmatis* (B). Protein samples were serially diluted 30-fold (A) or 10-fold (B). The mean apparent lag phase values are shown at each protein concentration tested (±SD, if this was greater than 3 h). Activity is represented schematically by the filled shapes, whose width is maximal at protein concentrations showing maximum activity (reduction of apparent lag phase, i.e. time to measurable turbidity in the Bioscreen C growth analyser). A dotted line indicates that activity is presumed to extend beyond the lowest or highest dilution tested. In controls with no added protein, the apparent lag phase was 216 ± 4 h (*M. luteus*) and 74 ± 4 h (*M. smegmatis*).

depended on the age of the inoculum. When actively growing cells were used, none of the proteins stimulated bacterial growth (representative data are shown for RpfC in Fig. 3F). However, in common with cells of both the virulent and the avirulent strains of *M. tuberculosis* (Sun and Zhang, 1999; Zhang *et al.*, 2001; Shleeva *et al.*, 2002), those of *M. bovis* (BCG) lose culturability during extended stationary phase. The activities of the five Rpf-like proteins of *M. bovis* (BCG) were therefore monitored using late stationary phase cells. As indicated in Fig. 3A–E, all five proteins were active; growth usually occurred after an appreciable lag, and was dependent on the provision of pM concentrations of any one of these five proteins. RpfA only showed activity at the lowest concentration tested (1.6 pM) (Fig. 3A), whereas the other proteins were active over the entire concentration range tested. The data summarized in Fig. 2A–B and Fig. 3A–E establish that the *rpf*-like genes of *M. tuberculosis* encode a family of growth factors with activities similar to that of *M. luteus* Rpf (Mukamolova *et al.*, 1998).

Rpf *expression in* M. smegmatis *stimulates bacterial growth*

To circumvent potential problems arising from the fact that the recombinant proteins are unstable and the proportion of biologically active molecules in different preparations of recombinant proteins is not known (see above), we examined the effect of Rpf expression *in vivo*. Plasmid pAGM0 (Mukamolova *et al.* 2002), expressing *rpf* under the control of the *M. smegmatis* amidase promoter (P_{ami}) (Parish *et al.*, 1997) was introduced into *M. smegmatis*. Using reverse transcriptase-polymerase chain reaction (RT-PCR), we verified that *rpf* is expressed soon after inoculation of the pAGM0-containing strain into fresh growth medium (data not shown). The appearance of Rpf in the culture supernatant was also demonstrable by Western blotting of samples taken when the cultures were in exponential phase (OD_{600} = 0.6). Using an inoculum grown to stationary phase overnight in NBE, bacteria were subjected to nutritional shift-down by inoculation at three different densities into Sauton's medium. At low inoculum densities (10^3 and 10^4 colony-forming units (cfu) ml^{-1}), the apparent lag phase was substantially reduced in the Rpf-expressing strain compared with that of the control containing the pAGH vector (Fig. 4A). This effect was observed in the presence of either kanamycin or hygromycin, used to select for plasmid maintenance and also in the absence of either antibiotic (data not shown). It was also observed in the absence of acetamide, consonant with the report that this promoter is partially active in the absence of inducer (Parish *et al.*, 1997). Growth stimulation was not apparent at a higher inoculum density (10^5 cfu ml^{-1}), nor was it ob-

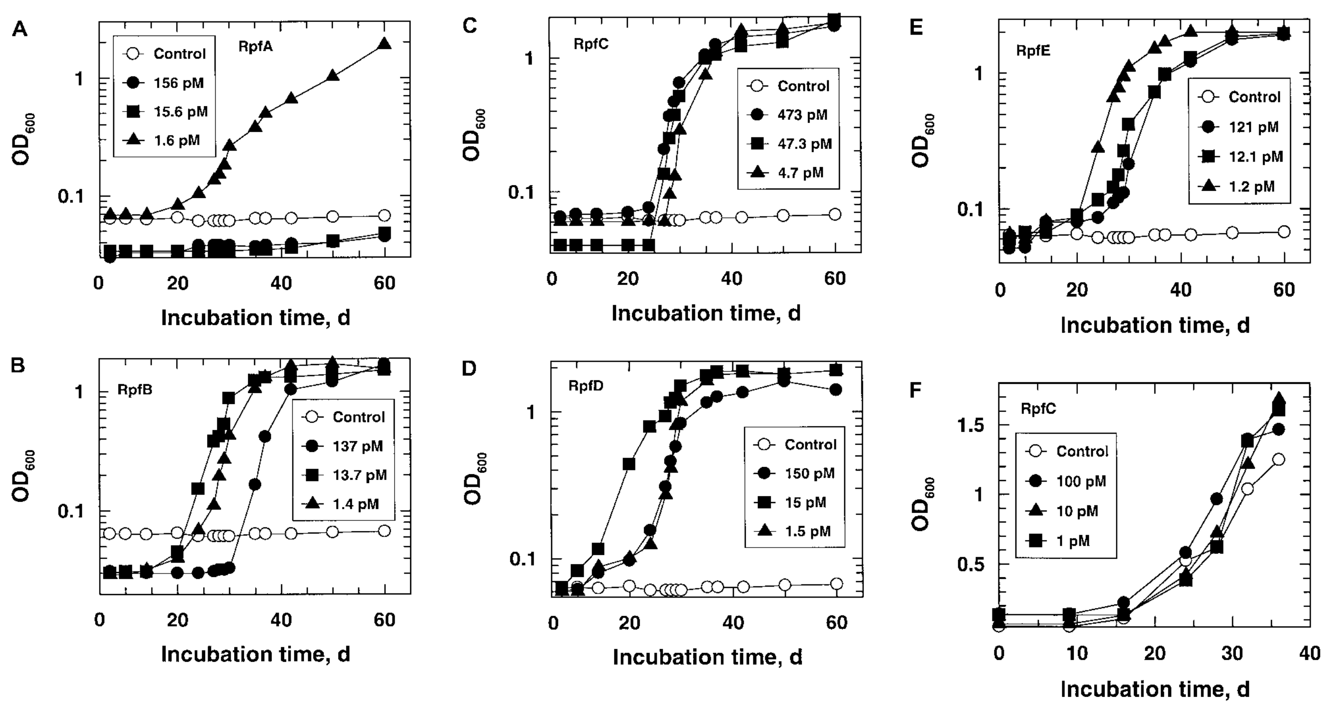

Fig. 3. The Rpf-like proteins of *M. tuberculosis* stimulate the growth of *M. bovis* (BCG). The responses of aged *M. bovis* (BCG) (5 month-old culture) to recombinant versions of RpfA (A), RpfB (B), RpfC (C), RpfD (D) and RpfE (E) are shown. The response of actively growing *M. bovis* (BCG) (inoculum was 100 cells ml^{-1} from late-logarithmic phase) to recombinant RpfC (F) is also shown. The OD_{600} of each culture was monitored with time.

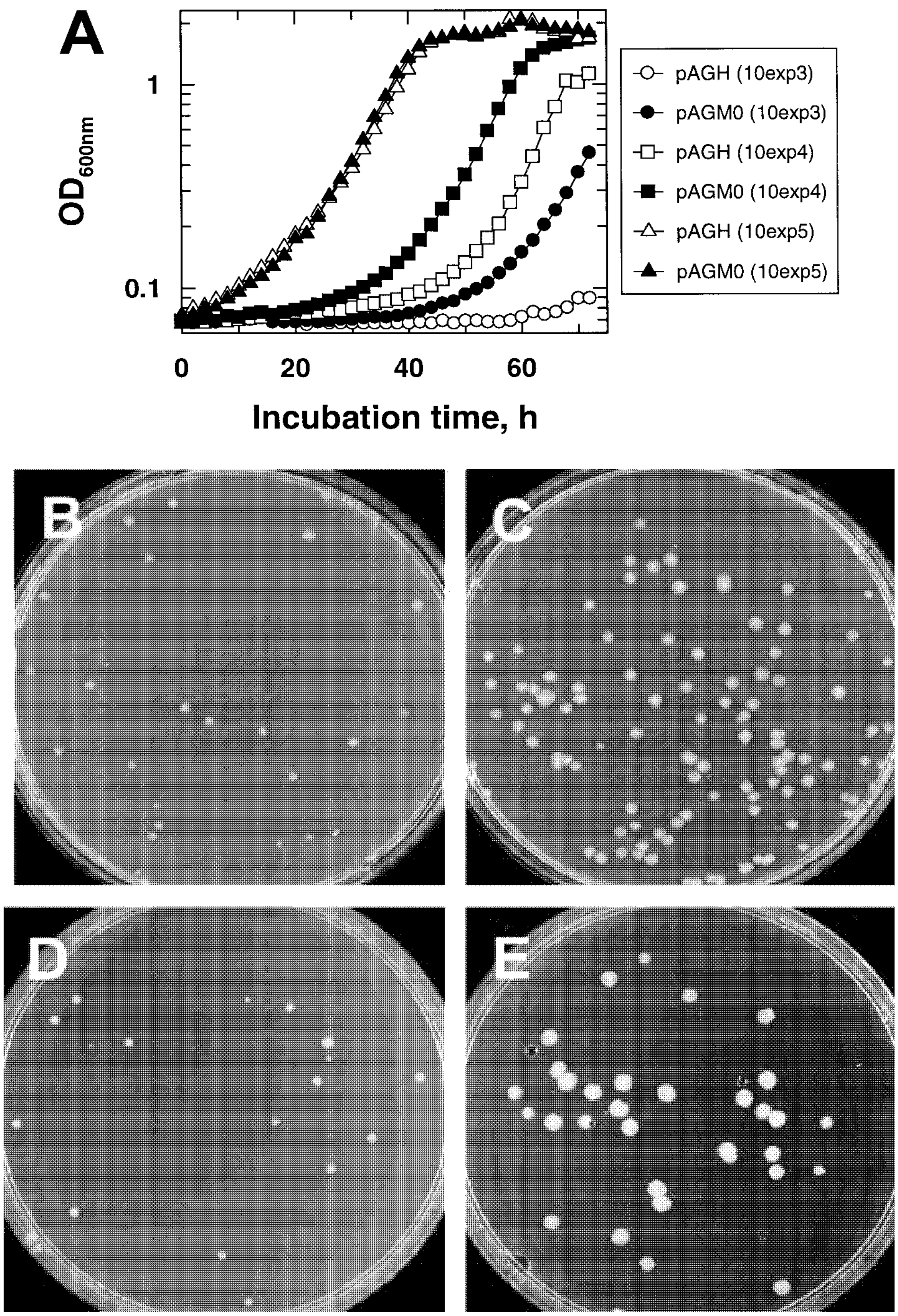

Fig. 4. Comparative growth kinetics (A) of *M. smegmatis* strains containing plasmids pAGH (vector) and pAGM0 (*rpf* expressed from P_{ami}) using Sauton's medium containing 0.05% Tween-80 and 5 μg kanamycin ml^{-1}. The number of cfu in the overnight cultures (adjusted to OD_{600} = 1.0, and used as inocula for this experiment) were 1.23 (±0.11) × 10^8 for the control containing pAGH, and 1.03 (±0.11) × 10^8 for the strain containing pAGM0. The remainder of the Figure shows colonies obtained from bacteria containing pAGH (B and D) or pAGM0 (C and E) 72 h post inoculation, on Sauton's medium containing ADC and either 50 μg hygromycin ml^{-1} (B and C) or 10 μg kanamycin ml^{-1} (D and E).

served in a strain harbouring pAGM1, in which P_{ami} generates a counter-transcript of *rpf*. The growth-stimulatory effect of *rpf* expression in *M. smegmatis* was also evident when comparing the sizes of colonies of strains harbouring pAGM0 (expressing *rpf*) and pAGH (vector control) obtained on agar-solidified Sauton's medium, both in the presence and absence of selective antibiotics (Fig. 4B–E and data not shown).

Expression of the five rpf*-like genes of* M. tuberculosis *and* M. bovis *(BCG)*

Messenger RNA corresponding to all five *rpf*-like genes was detected by RT-PCR using RNA isolated from cells of *M. tuberculosis* H37Rv and *M. bovis* (BCG) growing actively *in vitro* (Fig. 5A and B). Although only weak signals were obtained for RpfD (Rv2389c) in *M. bovis* (BCG) and RpfE (Rv2450c) in both organisms, expression of both genes has been detected by others in microarray experiments (Manganelli *et al.*, 2001; Sherman *et al.*, 2001). In contrast, we were unable to detect mRNA corresponding to any of these genes in RNA extracted from *M. bovis* (BCG) during stationary phase, nor from cells that had been starved for 5 months (data not shown). All five *rpf*-like genes are expressed in actively growing cells, whereas non-growing cells express them either at levels that are below the limit of detection, or not at all.

To monitor protein production, antibodies were raised against a histidine-tagged, truncated form of Rpf comprising residues A_{42}-L_{118}, i.e. the conserved 'Rpf domain' that is shared by all family members (Kell and Young, 2000), and purified by affinity chromatography (*Experimental procedures*). These antibodies reacted with recombinant versions of all five of the Rpf-like proteins of *M. tuberculosis* (Fig. 6C). They detected two bands in concentrated samples of supernatant obtained from exponentially growing cultures of *M. bovis* (BCG) (Fig. 6A) and *M. tuberculosis* (data not shown). Several bands were also detected in concentrated samples of supernatant obtained from exponentially growing cultures of *M. smegmatis* (Fig. 6B). Only the uppermost band was seen (and its apparent size was slightly reduced) if protease inhibitors were not present during isolation (see *Experimental procedures*). According to the available genome sequence information (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi), this organism potentially produces four Rpf-like proteins. These experiments confirm that Rpf-like proteins are detectable in the supernatant of actively growing cultures of *M. bovis* (BCG), *M. tuberculosis* and *M. smegmatis*.

Using confocal microscopy of *M. bovis* BCG that had been incubated with anti-truncated Rpf antibodies and a secondary, FITC-labelled antibody, Rpf-like protein(s) were detected on the bacterial cell surface. About 30% of the *M. bovis* (BCG) cells in early logarithmic phase cultures (8 days post inoculation) showed detectable fluorescence and some of them were much more strongly fluorescent than others (Fig. 6D and E). Fluorescence was abolished if recombinant Rpf was added to the bacteria at the same time as the primary anti-Rpf antibody. None of the cells in stationary phase cultures (6 weeks post inoculation) showed visible fluorescence (data not shown).

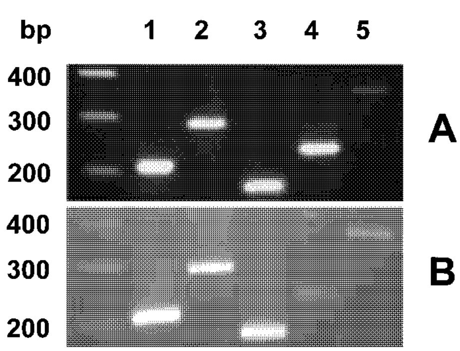

Fig. 5. The *rpf*-like genes of *M. tuberculosis* (A) and *M. bovis* (BCG) (B) are expressed *in vivo*. RT-PCR products obtained from *M. tuberculosis* H37Rv (A) and *M. bovis* (BCG) (B). RNA, using primer pairs specific for: 1, RpfA (209 bp); 2, RpfB (288 bp); 3, RpfC (180 bp); 4, RpfD (238 bp); 5, RpfE (357 bp) – sizes of expected products in parentheses. Markers of 200, 300 and 400 bp derived from a 100 bp ladder (Promega) are also shown.

Anti-Rpf antibodies inhibit bacterial growth

Given the accumulated evidence that members of the Rpf protein family stimulate bacterial growth from an extracytoplasmic location, we determined the effect of adding anti-Rpf antibodies to the culture medium. Immunoglobulins purified from immune serum partially inhibited the growth of the avirulent Academia strain of *M. tuberculosis*, whereas immunoglobulins purified from preimmune serum were without effect (Fig. 7A). Moreover, the inhibitory effect of immunoglobulins in the immune serum was overcome by the addition of Rpf. Similar growth inhibition of *M. bovis* (BCG) was observed using affinity-purified anti-Rpf antibodies. Inhibition was transient, resulting in delayed bacterial growth when using a large inoculum of late logarithmic cells (Fig. 7B). This was probably not a result of antibody degradation during the long incubation period, as anti-Rpf antibodies were detectable in the culture medium by Western blotting throughout the experiment (data not shown). There was more pronounced growth inhibition using a small inoculum of aged cells of *M. bovis* (BCG) (Fig. 7C). In all of these experiments, the addition of Rpf overcame the growth-inhibitory effect of the anti-Rpf antibodies. Indeed, in Fig. 7C the provision of Rpf abolished the short lag phase seen in the control.

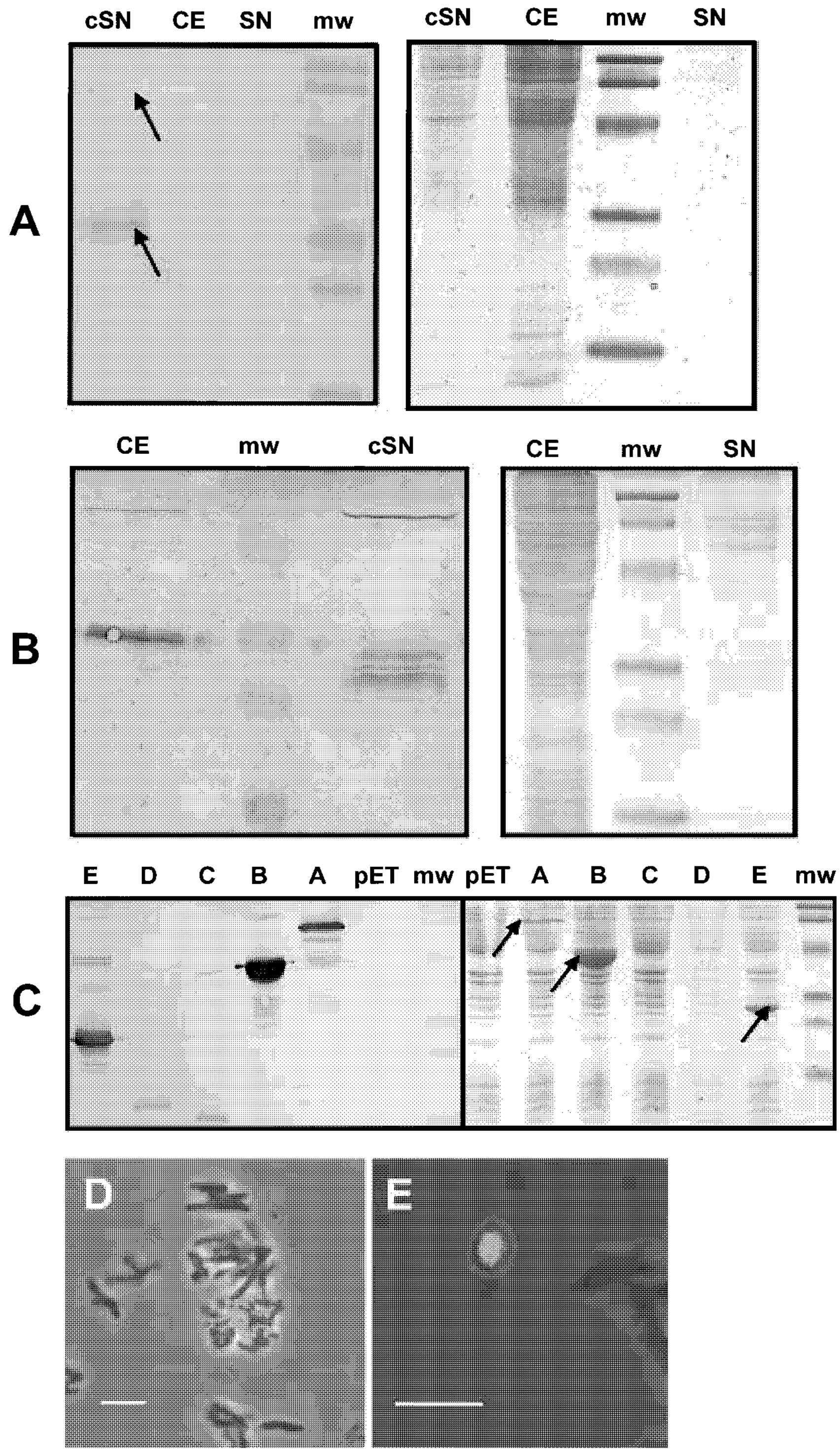

Fig. 6. Detection of Rpf-like proteins in culture supernatant from *M. bovis* BCG (A) and *M. smegmatis* (B) and on the surface of cells of *M. bovis* BCG (D and E). In A and B, samples of crude cell extract (CE), culture supernatant (SN) and proteins concentrated from culture supernatant using DEAE cellulose chromatography (cSN) are shown. C shows the recombinant Rpf-like proteins of *M. tuberculosis* in crude extracts of *E. coli* HMS174 following IPTG-induced expression (lanes A–E correspond to strains expressing RpfA-E; lanes labelled pET contain the pET-19b vector). In A–C, the left-hand panels show proteins detected by the rabbit anti-Rpf antibody and the right-hand panels show the corresponding Coomassie blue-stained gel. The size markers in A–C (98, 66, 45, 30, 22, 17 kDa), were from BioRad. The arrows in A indicate the positions of proteins detected on the immunoblot and those in C, indicate the positions of recombinant RpfA, B and E in the Coomassie blue-stained gel. C shows evidence of both aggregation and degradation of RpfA, RpfB and RpfE. D and E show confocal microscope images of fixed cells of *M. bovis* BCG from an exponential phase culture (8 days post inoculation) following incubation with anti-truncated Rpf antibodies and a secondary FITC-labelled antibody. E is a view of the pole of a fluorescent cell.

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Discussion

Information from the published genome sequence (Cole *et al.*, 1998) suggested that the *rpf*-like genes of *M. tuberculosis* encode a family of surface-located or secreted proteins. This was confirmed by the results reported here. One or more of these proteins was detected on the surface of actively growing bacteria and in concentrated samples of supernatants obtained from cultures of *M. tuberculosis*, the closely related organism, *M. bovis* (BCG), and *M. smegmatis* (Fig. 6A and B). Moreover, growth of *M. bovis* BCG was stimulated by recombinant versions of these Rpf-like proteins (Figs 2 and 3), and inhibited when anti-Rpf antibodies were incorporated into the culture medium (Fig. 7).

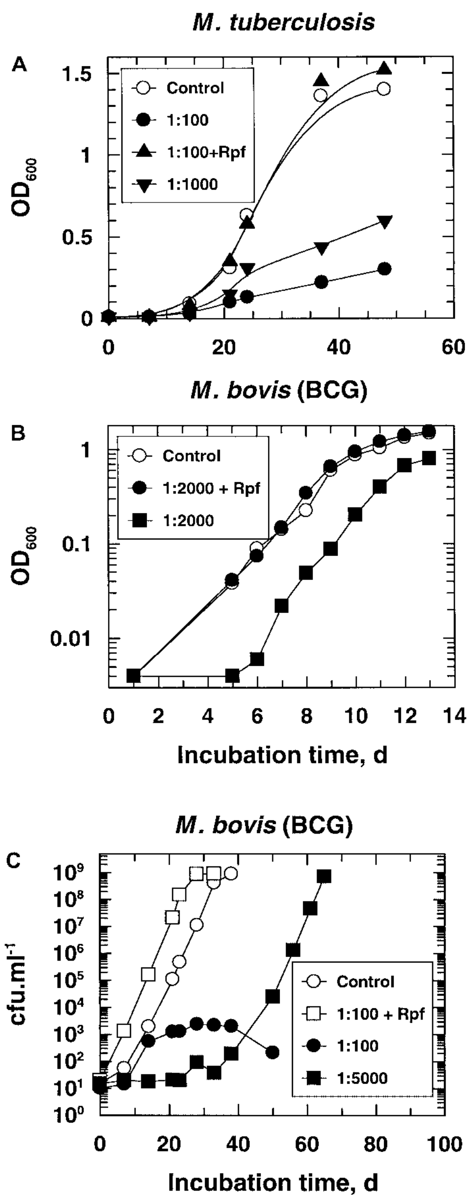

Fig. 7. Influence of anti-Rpf antibodies on bacterial growth. Cultures were inoculated with 10^5 cfu from a 2-month-old culture of *M. tuberculosis* (A), 10^6 cfu from a 2-week-old culture of *M. bovis* (BCG) (B) and 10^2 cfu from a 6-week-old culture of *M. bovis* BCG (C). The IgG fraction purified from immune and preimmune sera (A) and affinity-purified rabbit anti-Rpf antibodies (B and C) were used and Rpf (450 pM) was added as indicated. Growth was monitored by measuring the OD_{600} (A and B) or by plating (C).

The Rpf-like proteins of *M. tuberculosis* stimulated bacterial growth in laboratory culture at very low (pM) concentrations, which effectively excludes the possibility that they were simply being used as nutrients. The most potent proteins were active at concentrations equivalent to just a few molecules per cell (Fig. 2), consistent with the view that they act as growth factors. As was previously noted when *M. luteus* Rpf was tested against a panel of different organisms, the Rpf-like proteins of *M. tuberculosis* show cross-species activity. They stimulated the growth of the closely related, slow-growing organism, *M. bovis* (BCG) (Fig. 3) as well as that of two fast-growing organisms, *M. smegmatis* and *M. luteus* (Fig. 2). Moreover, expression of *rpf* in *M. smegmatis* also stimulated the growth of this organism in a minimal medium (Fig. 7), confirming the results obtained with exogenously added recombinant proteins. It is noteworthy that this represents the first demonstration of Rpf-mediated growth stimulation on a solid medium; when recombinant proteins are incorporated into molten agar or spread on the surface of agar plates they are not active (Kaprelyants *et al.*, 1994; Mukamolova *et al.*, 2002).

Reverse transcriptase–polymerase chain reaction showed that actively growing cells of both *M. tuberculosis* H37Rv and *M. bovis* (BCG) express all five of their *rpf*-like genes (Fig. 5) and Western blotting indicated that Rpf-like proteins are detectable in the culture medium (Fig. 6A). Continued cellular multiplication is assured by endogenous protein production; hence, the provision of extra molecules in the culture medium has little effect on bacterial growth (Fig. 3F and unpublished data). Moreover, some organisms appear to produce sufficient amounts of these growth factors to render them relatively insensitive to the growth-inhibitory effect of anti-Rpf antibodies (Fig. 7B) and to permit their detection on the cell surface (Fig. 6D–E). Non-growing bacteria do not produce these growth factors. As they remain in stationary phase, previously synthesized molecules gradually decay, and the bacteria become increasingly dependent on an exogenous supply in order to resume growth (Fig. 3A–E). A growth factor requirement is also detectable in cells of the avirulent Academia strain of *M. tuberculosis* that have persisted for several days within murine macrophages (Biketov *et al.*, 2000). Mycobacteria inoculated from late stationary phase, or even older cultures, either fail to grow, or grow only very slowly after an extended lag phase (Dubos and Davis, 1946; Aldovini *et al.*, 1993; Yuan *et al.*, 1996; Lim *et al.*, 1999; Sun and Zhang, 1999). The addi-

tion of Rpf-like growth factors to such cultures should aid the resumption of normal growth, which could potentially improve the diagnosis and quantification of mycobacterial infections.

To obtain 'non-culturable', Rpf-responsive cells of *M. bovis* (BCG) (see Fig. 3), bacteria were incubated in Sauton's medium in prolonged stationary phase, without exposure to the laboratory atmosphere. These conditions (except for oxygen depletion) were similar to those used previously to elicit dormancy in *M. luteus* (Mukamolova *et al.*, 1995). The avirulent H37Ra strain of *M. tuberculosis* can also enter a 'non-culturable' state after prolonged stationary phase, from which it can be resuscitated using culture supernatants (Sun and Zhang, 1999). Using an indirect, fluorescence-based assay that correlated with resuscitation, the authors initially reported that the active agent was a small MW, heat-stable compound that accumulated in early stationary phase. Subsequently (Zhang *et al.*, 2001), activity in early stationary phase supernatants was associated with phospholipids and an 8 kDa protein (Rv1174c). Three synthetic peptides representing different segments of this protein were active when added to bacteria at micromolar concentrations. In our experiments, in which minute (picomolar) concentrations of Rpf-like proteins permitted the growth of organisms that were otherwise non-culturable (Fig. 3A–E), we can effectively exclude any non-specific growth stimulation potentially arising from utilization of these molecules as nutrients.

A growth factor requirement can be artificially induced in growing cells of *M. luteus* by repeatedly washing them and inoculating them at low cell density (Mukamolova *et al.*, 1998; 1999). Rpf has a LysM lysin domain at its C-terminus, which is probably a peptidoglycan-binding module (Bateman and Bycroft, 2000). Washing presumably removes secreted Rpf molecules associated with the cell envelope. Attempts to induce a similar growth factor requirement in *M. tuberculosis* and *M. bovis* (BCG) by washing have proved unsuccessful (unpublished data). This is consistent with the observation that one or more of the Rpf-like proteins elaborated by these organisms, which tend to form extensive clumps, cords and pellicles, is probably membrane-bound (e.g. RpfB).

We suspect that the five different Rpf-like proteins of *M. tuberculosis* and *M. bovis* (BCG) fulfil subtly different, overlapping, biological functions. They may all act as autocrine signalling molecules, effectively stimulating the growth and multiplication of the cells that produce them. Free-living (planktonic) cells may use secreted proteins (e.g. RpfA) for paracrine, density dependent signalling (quorum sensing) (Fuqua *et al.*, 1996), whereas cells growing in close proximity may use envelope-associated proteins for juxtacrine signalling (Kaprelyants *et al.*, 1999). Dormant or injured cells (defined in Kell *et al.*, 1998) require an exogenous source of these proteins for their resuscitation (Mukamolova *et al.*, 1998; Biketov *et al.*, 2000). Further insights into their precise biological roles will require the construction and analysis of mutants harbouring null mutations.

The growth-promoting effects of these proteins appear to result from their specific interaction with a component of the bacterial cell envelope. Two lines of evidence suggest that they bind to specific receptors. One is their extreme potency. The other is that they have an optimum concentration for biological activity, above which they are either inactive or even inhibitory (Mukamolova *et al.*, 1998; 2002). Evidence of this nature has previously been adduced in studies of hormone–receptor and ligand–receptor interactions (Franklin, 1980; Gero, 1983).

When mycobacteria experience an extended stationary phase *in vitro* they often lose culturability (Sun and Zhang, 1999; Zhang *et al.*, 2001; Shleeva *et al.*, 2002). They may possibly enter a state of Rpf-responsive dormancy, akin to that observed when *M. luteus* experiences prolonged stationary phase (Kaprelyants and Kell, 1993; Kaprelyants *et al.*, 1993). Cells that have been starved *in vitro* may be physiologically similar to bacteria that persist *in vivo*, in patients harbouring a latent *M. tuberculosis* infection (Parrish *et al.*, 1998). Persisting organisms are detectable (de Wit *et al.*, 1995; Hernandez-Pando *et al.*, 2000; Pai *et al.*, 2000), but cannot be cultured using conventional methods (plating or inoculation into liquid media) (Wayne, 1960, 1994;McCune *et al.*, 1966; de Wit *et al.*, 1995). It is consequently possible that (a lack of) Rpf-like growth factors contributes a microbiological component to the phenomenon of mycobacterial persistence in latent infections. The results reported here indicate that the Rpf family of growth factors may provide interesting opportunities for preventing and treating mycobacterial infections.

Experimental procedures

Organisms and media

Micrococcus luteus NCIMB 13267 ('Fleming strain 2665') was grown in LMM (Kaprelyants and Kell, 1993) at 30°C in conical flasks on an orbital shaker. *Mycobacterium bovis* (BCG) was obtained from the Central Institute for Scientific Research on Tuberculosis, Moscow, Russia. Cultures (3 ml) were grown at 37°C in 10 ml tubes with tightly fitting screw caps without shaking in Sauton's medium supplemented with ADC (Connell, 1994) and 0.05% Tween 80. *Mycobacterium smegmatis* mc^{2}155 was grown aerobically at 37°C in Sauton's medium.

Cell viability by plating

Dilutions in LMM (*M. luteus*) and Sauton's medium (*M. smegmatis*) were plated in triplicate on agar-solidified plates containing nutrient broth E (NBE – LabM) and incubated for 3 days at 30°C for *M. luteus*, or 37°C for *M. smegmatis*.

Dilutions of *M. bovis* (BCG) were made in Sauton's medium supplemented with 0.05% Tween 80, spread on agar-solidified Sauton's medium supplemented with ADC (Connell, 1994) and incubated at 37°C for 3–4 weeks. Suspensions of all organisms were passed 10 times through a 23-gauge needle to break up loose cell aggregates before dilution.

Bacterial growth kinetics

For the experiment shown in Fig. 4A, *M. smegmatis* strains containing pAGH or pAGM0 were grown overnight to stationary phase in NBE containing 20 μg hygromycin ml^{-1}. Under these conditions clumping is minimized. The cultures, which reached a final OD_{600} of 2.1 (pAGH) and 2.4 (pAGM0), were diluted with Sauton's medium to an OD_{600} of 1.0 and a further reduction in clumping was obtained by passing them ten times through a 23-gauge syringe needle. A sample was taken for cfu determination. Bacteria were then serially diluted and inoculated at three different densities into Sauton's medium (nutritional shift-down) supplemented with 0.05% Tween-80 in Bioscreen plates (five replicates for each strain at each density). Growth was at 37°C with constant shaking in a Bioscreen C optical growth analyser (Laboratory-systems, Finland). The data shown are the averages of readings from the five replicate cultures. The standard deviations on these measurements are smaller than the data points on the Figure. Similarly treated bacteria were used to inoculate the plates shown in Fig. 4B–E.

Production of recombinant proteins

Recombinant Rpf was obtained as previously described (Mukamolova *et al.*, 1998). The five *rpf*-like genes from *M. tuberculosis* were amplified from H37Rv DNA using the following primer pairs (*Eco*RI, *Nco*I, *Nde*I and *Bam*HI restriction sites introduced for cloning purposes are in italics): Rv0867F (CCA*GAATTCATATG*GCTCAGGCGACCGCGGCCACC) + Rv0867R (TGGC*GGATCC*TATCAGCCGATGACGTACGCTG); Rv1009F (5′-GTGG*CCATGG*G*CATATG*GCAAGCAAAACGGTGACGTTGA3′) + Rv1009R (5′CAGCC*GGATCC*TCAGCGCGCACCCGCT-3′); Rv1884F (5′-TCCT*GAATTCATATG*GGTCCCAGCCCGAACTGG-3′) + Rv1884R (5′-CATG*GGATCC*GTCAGCGCGGAATACTTG-3′); Rv2389F (5′-ATCA*GAATTCATATG*GACGACATCGATTGGGACGC-3′) + Rv2389R (5′-CGCA*GGATCC*CCTCAATCGTCCCTGCTCC-3′); Rv2450F (5′-TGGA*GAATTCATATG*GACGACGCGGGCTTGGA-3′) + Rv2450R (5′-TCTT*GGATCC*TATCAGCCGCGGCGGCCGCA-3′). Amplification produced derivatives of each gene lacking their 5′ ends, predicted to encode signal sequences or N-terminal *trans*-membrane helices/anchors (http://www.cbs.dtu.dk/services/SignalP/and http://www.cbs.dtu.dk/services/TMHMM-1.0/). In the case of RpfB (Rv1009), an additional $C_{24}S$ substitution was introduced. The truncated form of *rpf* was amplified from a previously cloned (Mukamolova *et al.*, 1998) 1375 bp segment of *M. luteus* DNA using primers TR1 (5′-GTCA*GAATTCATATG*GCCACCGTGGACACCTG-3′) + TR2 (5′-TGAC*GGATCC*TATTACAGCTTCTGCGAGCACAG-3′). Polymerase chain reaction products were first established in *E. coli* XL-2 blue as *Eco*RI-*Bam*HI or *Nco*I-*Bam*HI fragments in pMTL20 (Chambers *et al.*, 1988) and their sequences verified. They were then cloned as *Nde*I-*Bam*HI fragments in pET19b (Novagen) and re-established in *E. coli* XL-2 blue. The polyhistidine-tagged proteins were expressed in *E. coli* HMS174 (DE3) and purified essentially as described (Mukamolova *et al.*, 1998) to single band purity by SDS-PAGE. The appearance of cell extracts is shown in Fig. 6C, from which it is clear that these proteins are subject to both aggregation and degradation when expressed as recombinant derivatives in *E. coli*. Anti-His tag antibodies (data not shown) also detect the various bands detected by the anti-Rpf antibodies (Fig. 6C). Except in the case of RpfB, additional purification was by Mono Q chromatography. RpfC (Rv1884c) and RpfD (Rv2389c) eluted as single peaks whereas RpfA (Rv0867c) and RpfE (Rv2450c) and *M. luteus* Rpf (full length and truncated form) were present in several fractions (revealed by immunoblotting) one of which only, was biologically active. The active fraction was used for experiments. Freshly isolated proteins (usually same day or next day) were used in all experiments, as activity is substantially reduced (1 log) during storage for 1 week at 20°C in 50% glycerol.

Activity assay

Growth of *M. luteus* and *M. smegmatis* was monitored in a Bioscreen C growth analyser (Labsystems, Finland) using a 600 nm filter. Freshly prepared recombinant proteins diluted 1 : 100 in the appropriate growth medium were sterilised by filtration (0.22 μm, Gelman) and then serially diluted in growth medium either 30- or 10-fold, for assays with *M. luteus* and *M. smegmatis* respectively. After breaking up aggregates (see above), late log cultures (OD_{600} = 3.5–4.0 for *M. luteus*; OD_{600} = 3.0–3.5 for *M. smegmatis*) were serially diluted using growth medium. Samples (5 μl) of each dilution (five to ten replicates) were added to wells containing medium (200 μl), together with serially diluted protein. Incubation was at 30°C (*M. luteus*) or 37°C (*M. smegmatis*) with continuous shaking on the high setting. Measurements were taken hourly for 240 h. *Mycobacterium bovis* (BCG) bioassays were in tubes (see above) inoculated with cells (10^2–10^6 cells per ml) from cultures of different ages (10 days–5 month). Incubation was at 37°C without shaking and growth was monitored by direct measurement (OD_{600}) of the tube cultures or by plating.

Antibody purification and Western blotting

Rabbits were immunized three times at 3-week intervals by subcutaneous injection with 1 ml of a 50% (v/v) mixture of Rpf (1 mg ml^{-1} in water) and incomplete Freund's adjuvant (Sigma). Serum was collected 10 days after the last immunization and antibodies were purified by affinity chromatography [Rpf conjugated to CNBr-activated Sepharose 4B, (Sigma)]. Sheep antibodies to the truncated form of Rpf were obtained commercially (Micropharm, Newcastle Emlyn, UK) and purified as above. Both types of antibodies also detected recombinant versions of all five Rpf-like proteins of *M. tuberculosis*, as well as various aggregated forms and degradation products (see Fig. 6C for reactions to rabbit anti-Rpf antibody). The rabbit antibodies were used for the experiments

shown in Fig. 7, except the experiment in Fig. 7A, for which immunoglobulins purified from preimmune and immune serum using G-protein Sepharose (Pharmacia) were used.

For Western blotting, 1 ml of a 3-week-old culture of *M. bovis* (BCG) Russian strain, grown in Sauton's medium supplemented with ADC and 0.05% Tween-80, was washed twice with Sauton's medium to remove traces of ADC and inoculated into 100 ml Sauton's medium in a 500 ml flask. Growth was for 3 weeks at 37°C without shaking. For *M. smegmatis*, growth was for 16 h (overnight) with shaking in Sauton's medium (lacking ADC and Tween-80). A protease inhibitor cocktail was added to the cultures 30 min before harvesting and incorporated into all buffer solutions, according to the manufacturer's instructions (Roche). After centrifugation, culture supernatants were filtered (0.22μm) and proteins present were concentrated using DEAE-Sepharose 6B and CM cellulose column chromatography. The DEAE-Sepharose 6B fast flow column, equilibrated with buffer A (20 mM TrisHCl, pH 7.5; 20 mM KCl, 1 mM EDTA, 1 mM DTT) bound all detectable Rpf-like proteins. The column was washed with 5 × vol buffer A and eluted with 3 × vol buffer B (buffer A containing 1 M NaCl). Samples (1 ml) of the eluate were precipitated with 10% TCA, washed twice with acetone, dried, reconstituted in 20 μl loading buffer and used for SDS gel electrophoresis/blotting. Pre-stained size standards were from BioRad (cat no. 161–0305). For the experiments shown in Fig. 6, rabbit anti-Rpf antibodies were used; similar data were obtained with the sheep anti-truncated Rpf antibodies.

Confocal microscopy

Cells from early logarithmic phase ($OD_{600} = 0.25$) grown in Sauton's medium supplemented with ADC and 0.05% Tween-80, were centrifuged, washed with PBS and fixed in 0.1% glutaraldehyde in PBS for 30 min at 37°C. After washing with PBS, cells were incubated in PBS containing 5% BSA for 30 min at 37°C with shaking, before treatment with sheep anti-truncated Rpf antibodies at 1 : 1000 dilution in PBS containing 2% BSA. After incubation for 1 h at 37°C with shaking, cells were washed three times with PBS, containing 0.2% Tween-80. The bacteria were then incubated in PBS, containing 2% BSA and FITC-conjugated donkey anti-sheep IgG antibodies at a 1 : 500 dilution (Sigma, cat. no. F7634). After washing (as described above) cells were examined using a BioRad MRC1024ES confocal microscope with excitation at 488 nm (100 mW argon laser).

Effect of anti-Rpf antibodies on bacterial growth

Mycobacterium bovis (BCG) (10^2 cfu from a 2-week-old culture or 10^6 cfu from a 6-week-old culture) and *M. tuberculosis* Academia strain (10^5 cfu from a 2-month-old culture) were inoculated into 3 ml Sauton's medium supplemented with ADC and 0.05% Tween-80. Rabbit anti-Rpf antibodies were added and growth was monitored by measuring the OD_{600}. For the experiment shown in Fig. 7C, a 20 ml culture was established in a 100 ml conical flask without shaking and growth was monitored by plating on agar-solidified Sauton's medium supplemented with ADC.

Expression of rpf *in* M. smegmatis

The *rpf* gene was introduced into *M. smegmatis* under the control of the P_{ami} promoter (Parish *et al.*, 1997) in plasmid pAGM0. The construction of this plasmid and the vector, pAGH, from which it is derived, was described by (Mukamolova *et al.* (2002). Plasmid pAGM1 is similar to pAGM0 except that P_{ami} generates a counter-transcript of *rpf*.

Reverse transcriptase-PCR

Specific primer pairs were designed for each of the five *rpf*-like genes of *M. tuberculosis* (the corresponding regions of the cognate genes in *M. bovis* are identical): RT0867F (5′–TATGAGTGGACGCCACCGTAA–3′) + RT0867R(5′–ACTGC AAGCCACCGAGGTAAC3′); RT1009F (5′AGGACCCGGAG ATGAACATGA3′) + RT1009R (5′GCACACCACCGTAATAC CCGT–3′); RT1884F (5′–GCTTCTCGGGAACAACAAATC–3′) + RT1884R (5′–CGGAATACTTGCCTGAATGCC–3′); RT 2389F (5′–GCTATGACACCGGGTTTGCTT–3′) + RT2389R (5′–GCAGACCACCGTATAACCCGT–3′); RT2450F (5′–GT TGAAGAACGCCCGTACGAC–3′) + RT2450R (5′–TTACCG GTGTTGATCGACCAG–3′). RNA was prepared from 1 ml culture samples of *M. bovis* (BCG) ($OD_{600} = 0.3$) using the RNeasy Mini Kit (Qiagen) and treated twice or three times with 10 U of RNAase-free DNAase I (Roche) for 30 min. The RNA from exponentially growing cells of *M. tuberculosis* H37Rv was generously provided by P. Butcher and J. Mangan. Reverse transcription (25 μl) reactions contained 2 μg RNA, 1 μg of the relevant reverse primer, 40 U RNAsin ribonuclease inhibitor (Promega) and 30 U AMV reverse transcriptase (Promega). Reactions (1 h) were performed at 60°C in the presence of 10% DMSO for templates containing 71–74% G + C (RpfA, RpfC and RpfD) and 1 M betaine for templates containing 83–85% G + C (RpfB and RpfE). Reactions were terminated by incubation at 75°C for 5 min. Control reactions, lacking AMV reverse transcriptase were performed simultaneously. For PCR reactions, 2 μl samples of the RT reaction products were used as template in the presence of both primers. Samples were denatured for 5 min at 94°C followed by a single cycle of 30 s at 94°C, 30 s at 52°C, 60 s at 72°C and then 29 cycles of 30 s at 94°C, 60 s at 72°C. No PCR product was produced in any of the control reactions in which reverse transcriptase had been omitted from the previous step, nor when a treatment with RNAase preceded the initial reverse transcription step.

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